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(71) Applicant *(for all designated States except US)*: CENTRAL BLOOD LABORATORIES AUTHORITY [GB/GB]; The Crest, Dagger Lane, Elstree, Borehamwood, Hertfordshire WD6 3AU (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : JUDSON, Philip, Allan [GB/GB]; 66 Lake Road, Henleaze, Bristol BS10 5JF (GB). SMYTHE, Jonathan, Spencer [GB/GB]; 55 Sefton Park Road, St. Andrews, Bristol BS7 9AN (GB).

(74) Agents: HOLMES, Michael, John et al.; Frank B. Dehn & Co., Imperial House, 15-19 Kingsway, London WC2B 6UZ (GB).

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(54) Title: MODIFIED ANTIBODIES

(57) Abstract

The present invention relates to modified forms of antibodies having specificities against polysaccharide antigenic determinants which antibodies in the native form produce a precipitate on storage at 4 °C in aqueous solution said modified antibodies carrying modified glycosyl groups and on storage in aqueous solution at 4 °C producing substantially no precipitate.

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Modified antibodies

This invention relates to modified antibodies, particularly monoclonal antibodies, and a method for their production.

For many uses, for example in the determination of the ABO blood groups of patients and blood donors, it is convenient to prepare standard aqueous solutions of monoclonal antibodies having specificity against antigens of interest. However, in the case of some monoclonal antibodies having specificity against polysaccharide antigens, it is found that such standard aqueous solutions on storage at 4°C, form a precipitate which appears to be a cross-linked form of the antibody.

Monoclonal antibodies are produced by cell-lines as populations of closely related structures in which the protein portion is substantially constant but is glycosylated to varying degrees. Within cells, the process of the biosynthesis of N-linked glycans of glycoproteins such as immunoglobulins begins on a large carrier lipid molecule dolichol pyrophosphate. Monosaccharides are added to this precursor and the final product is a dolichol pyrophosphate oligosaccharide containing 3 glucose, 9 mannose and 2 N-acetylglucosamine residues (Glc3 Man9 GlcNAc2) in the endoplasmic reticulum. This Glc3 Man9 GlcNAc2 oligosaccharide is enzymatically transferred from dolichol pyrophosphate to an asparagine residue on the nascent polypeptide to form the glycoprotein precursor. The oligosaccharide then undergoes a complex series of processing reactions in the endoplasmic reticulum and golgi apparatus of the cell, involving first the trimming of the Glc3 Man9 GlcNAc2 by glycosidases, and then rebuilding of the oligosaccharide by glycosyltransferases adding specific monosaccharides

until the final N-glycan is completed. Trimming by glycosidases must be completed before further processing by the glycosyltransferases can occur.

In normal biosynthesis, a range of different N-glycan groups may be attached to the antibody. Where the antigen binding sites of the antibody are specific to a polysaccharide antigen, the possibility arises of cross-linking between the antigen binding sites and any regions of the N-glycan groups which are wholly or partly homologous with the antigen against which the antibody is specific. Such cross-linking is believed to be the mechanism by which certain antibodies specific against polysaccharides form unwanted precipitates on storage.

In particular, the trisaccharide Gal α 1-3 (Fuc α 1-2) Gal β 1- is the minimal determinant structure of the B blood group antigen (Clausen & Hakomori, 1989). This can be the terminal carbohydrate sequence of glycolipids, or O -glycans and/or N - linked glycans of glycoproteins. Mouse IgM has N - glycans on 5 different amino acid positions in the molecule. Studies of the carbohydrate structures of the mouse IgM myeloma protein MOPC104E (Anderson et al, 1985) showed that there was heterogeneity in the glycosylation of these glycans and that some of the N-linked oligosaccharide chains at one of the five glycosylation sites on the molecule were terminated by alpha 1 - 3 linked galactose, as in the B antigen structue. Thus these oligosaccharides may express B-antigen like reactivity.

We have found that by modifying the N-glycan residues of such monoclonal antibodies it is possible to reduce or eliminate the formation of an unwanted precipitate on storage at low temperatures. This may be achieved by biosynthesis of the antibodies in the presence of an inhibitor or inhibitors of glycoprotein processing and/or by subjecting the antibody after synthesis to the action of a glycosidase or

glycosidases, whereby any N-glycan groups attached to the antibody no longer include regions interacting with the antigen-binding regions.

The invention thus provides modified forms of antibodies having specificities against polysaccharide antigenic determinants which antibodies in the native form produce a precipitate on storage at 4°C in aqueous solution, said modified antibodies carrying modified glycosyl groups and on storage in aqueous solution at 4°C producing substantially no precipitate.

The invention is applicable to both monoclonal and polyclonal antibodies.

As indicated above, the antibodies concerned are ones in which at least some species of the native unmodified antibodies carry glycosyl groups which cross-link with the binding sites specific to the said polysaccharide antigenic determinants, the modified glycoside groups being such that they no longer cross-link with the above binding sites.

The monoclonal antibodies concerned are most commonly IgM immunoglobulins and as these carry multiple N-glycan groupings they are particularly susceptible to the cross-linking phenomenon.

As indicated above, anti-B monoclonal antibodies are active against a trisaccharide antigen on the red cells and further frequently possess N-glycan groupings having at least one point of homology with the antigen. For use in direct agglutination tests anti-B antibodies are normally IgM and thus carry several N-glycan groups capable of cross-linking.

The invention thus particularly provides IgM anti-B monoclonal antibodies having modified glycosyl groups and which do not produce a precipitate on storage at 4°C in aqueous solution. Such modified antibodies are particularly useful in blood typing.

It appears that certain sub-populations of the native unmodified monoclonal antibodies are free from

glycosyl groups which cross-link with the binding sites or otherwise cause formation of a precipitate. However, the invention provides, for the first time, modified forms of antibody preparations having specificities against polysaccharide antigenic determinants and in the native form producing a precipitate on storage at 4°C in aqueous solution, comprising a mixture of antibodies each having the same polypeptide moiety but carrying different glycosyl groups, the mixture in the native form producing a precipitate at 4°C in aqueous solution, the modified antibodies carrying modified glycosyl groups whereby on storage in aqueous solution at 4°C the mixture does not form a precipitate. In particular, the invention provides IgM anti-B antibody preparations having modified glycosyl groups wherein the preparation as a whole does not produce a precipitate on storage at 4°C in aqueous solution.

More especially, the invention provides antibodies having specificities against polysaccharide antigenic determinants, especially IgM anti-B monoclonal antibodies, wherein, in place of native glycosyl groups having regions of homology with said determinants, there are glycosyl groups of a different structure lacking said regions of homology.

The invention further provides a process for the production of modified monoclonal antibodies according to the invention in which a cell line producing said monoclonal antibodies in unmodified form is cultured in the presence of one or more inhibitors of glycoprotein processing and/or the antibodies synthesised by the cell line are subjected to the action of one or more glycosidases.

Inhibitors of glycoprotein processing glycosidases are known, and can be added to cell cultures without being cytotoxic. The cells thus treated are inhibited from making glycoproteins with normal complex type N glycans, but instead make abnormally glycosylated

glycoproteins of the high mannose type. Such high mannose glycans can be removed from the remainder of the molecule using the endoglycosidase Endo H. These abnormally glycosylated glycoproteins appear to support most of the normal cellular functions, since at low doses the inhibitors are not cytotoxic. Hashim and Cushley (1988) have shown that immunoglobulin secretion from hybridoma cell lines can proceed in the absence of N-linked oligosaccharide processing, and that the immunoglobulins have an abnormal structure.

Inhibitors of glycoprotein processing include castanospermine, deoxymannojirimycin (DMM), swainsonine and deoxynorjirimycin.

An alternative embodiment of the invention is the treatment of the monoclonal or polyclonal antibodies with glycosidases, to alter the nature of the N-glycans of the antibody. According to a particular aspect such glycosidase enzymes may be added to hybridoma cell cultures to alter the nature of the N-glycans of the monoclonal antibody molecules as they are secreted into the culture medium. Suitable glycosidases include in particular, alpha-galactosidase, e.g. coffee bean alphagalactosidase. According to another aspect the glycosidases may be used to modify isolated monoclonal or polyclonal antibodies.

The cell-line producing the antibodies may be a hybridoma or other immortalised cell line capable of cultivation in vitro. Murine hybridoma cell lines are in common use.

The culture conditions will be essentially the same as those used conventionally; thus the medium will contain assimilable sources of nitrogen, carbon and energy, as well as nutrients, vitamins and trace minerals. Dulbecco's modified Eagle's medium containing FCS is particularly effective. The concentration of glycoprotein processing inhibitor will commonly be in the range 2 to 50 μg/ml. DMM is effective at lower

doses than castanospermine, e.g. 2 to 15 μg/ml being optimal at about 12 μg/ml. Where a glycosidase is added to the medium, the concentration is advantageously in the range 1 to 10 μg/ml, for example about 5μg/ml.

Where a glycosidase such as an alpha galactosidase is used to modify isolated antibodies, the enzyme concentration will normally be in the range recommended by the manufacturer of the enzyme for the treatment of isolated glycoproteins. After production by cell culture, the antibody may be isolated from the supernatant, for example by conventional means such as precipitation, e.g. using high concentrations of an electrolyte such as ammonium sulphate. However, it may be convenient for many purposes to use the culture supernatant directly, and the invention extends to such supernatants containing the modified antibodies according to the invention together, interialia, with one or more inhibitors of glycoprotein processing and/or glycosidase enzymes.

The following Example is given by way of illustration:-

EXAMPLE

A. Materials and Methods

The cell line studied was NBTS/BRIC 30, an IgM anti-B secreting murine hybridoma cell line. It was generated by standard hybridoma techniques (eg Galfre et al 1977, Parsons et al 1982) of fusing together spleen cells from immunised mice with murine myeloma cells, in this instance from the NS-1 myeloma cell line.

In the fusion experiment which gave rise to BRIC 30, the mouse was immunised intraperitoneally with human erythrocytes of blood group B. The mouse was given 13 immunisations each of 100μl 50% suspension of washed group B erythrocytes at approximately weekly intervals.

The spleen cells were collected and fusion performed 3 days after the final immunisation. Secreting hybrids were detected by haemagglutination testing with human erythrocytes of various blood groups. Full details of immunisation, fusion and screening procedures are given in S F Parsons (1984).

The NBTS/BRIC 30 cell line was normally maintained in Dulbecco's modified Eagle's medium + 5% FCS (DF). Oligosaccharide processing inhibitors deoxymannojirmycin (DMM) and castanospermine were obtained from BCL (Lewes, UK). Stock solutions at 5-10mg/ml were prepared in DF and stored frozen. Cells were grown in 2ml culture wells or in 10ml volumes in T25 flasks at a seeding density of $1x10^5$/ml, with or without the addition of the appropriate inhibitor.

Precipitation of the immunoglubulin was quantitated using an ELISA assay for mouse IgM. Culture supernatants were clarified by centrifugation and sodium azide added to 0.1% w/v. Aliquots of 0.75ml were stored at 4°C for 7 days, and control aliquots were stored at room temperature for the same time. The test aliquots were centrifuged at 13000 rpm for 5min. at 4°C and the supernatant removed for assay in parallel with the room temperature stored sample. The concentration of IgM in each was determined by ELISA. The percentage IgM in the supernatant after storage was calculated by:

$$\% \text{ soluble} = \frac{[\text{IgM}] \text{ in } 4°\text{C stored sample}}{[\text{IgM}] \text{ in room temp. stored sample}} \times 100$$

Endo H treatment was based on the method described by Hashim & Cushley (1988). Culture supernatant samples were diluted in DF to 25μg/ml. 0.5ml was precipitated with 0.33ml saturated ammonium sulphate (40%) for 3 hr

at room temperature. The precipitate was washed in 40% ammonium sulphate and redissolved in 50µl 50mM sodium acetate, 0.5mM PMSF 0.1mg/ml SDS, pH 5.5. This was boiled for 2 min., cooled, and 5mU Endo H (BCL) added. The reaction was incubated at 37°C for 72hr. The reaction was stopped by adding 50µl reduced gel electrophoresis buffer. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was as described (Laemmli, 1970). Proteins were transferred to PVDF membranes (Millipore) by Western blotting, and the IgM µ chains visualised using peroxidase conjugated goat anti-mouse IgM µ chain (Sigma, Poole).

Alpha galactosidase was used to treat BRIC 30 IgM partially purified by 40% ammonium sulphate precipitation. BRIC 30 IgM (approximately 5mg/ml in 50mM sodium acetate buffer pH 6.5, 1mM PMSF), 0.5ml was treated with 0.5mg coffee bean alpha galactosidase (BCL) for 72hr at 37°C. The reaction mixture was diluted 1 in 5 with PBS containing 1% BSA and precipitation assayed as described above.

B. Precipitation of BRIC 30

Fresh BRIC 30 culture supernatant was stored at 4°C for 72hr, centrifuged at 13000rpm, 5min. and the supernatant collected. This supernatant was stored at 4°C for a further 24hr and the supernatant and pellet collected as above. The pellets were redissolved in the original volume of DF and the IgM concentrations determined by ELISA. The results (Table 1) show that after the initial cryoprecipitation, very little of the soluble IgM could be precipitated by further storage at 4°C. Thus there appear to be two populations of IgM anti-B molecules, one subset precipitable at 4°C, the other not. The proportion precipitable was about 80%.

The effect of the initial concentration of the IgM on the amount soluble after storage at 4°C was determined. Dilutions of BRIC 30 supernatant in DF were prepared and allowed to precipitate at 4°C for 24hr. The amount of IgM in the supernatant after centrifugation was compared to the same dilution stored at room temperature (Fig. 1). There was found to be a direct relationship between the amount of IgM precipitated and the initial concentration of IgM.

C. Dose - Dependent effects of glycoprotein processing inhibitors.

Two inhibitors of oligosaccharide processing were studied. DMM is an inhibitor of Golgi alpha - mannosidase 1 (Fuhrmann et al, 1984), and castanospermine is an inhibitor of endoplasmic reticulum glucosidases 1 and 2 (Sasak et al, 1985).

Various amounts of inhibitor stock solutions were added to 2ml cultures of BRIC 30. After 24hr the cell free supernatant was largely removed and replaced by fresh medium containing the same concentration of inhibitor. The cells were allowed to grow to death (about 5 days) and the % soluble IgM determined.

In the absence of inhibitors only 20% - 30% of BRIC 30 remained soluble after 1 wk. storage at 4°C. There was a dose - related increase in the amount of soluble IgM with both inhibitors (Fig. 2). The effect of DMM rapidly reached a plateau at around 5-10 μg/ml, whereas higher concentrations of castanospermine were required to give the maximum inhibitory effect.

At higher concentrations, castanospermine but not DMM affected the amount of total and soluble IgM produced (Fig. 3). Concentrations of DMM around 12μg/ml

gave optimal yields of soluble IgM.

Rates of growth in the presence of inhibitors were also examined. In 10ml cultures grown continuously over a 2 wk. period monitored with daily cell counts, the inhibitors DMM (to 25μg/ml) and castanospermine (to 50μg/ml) had no effect on the rate of cell growth (data not shown).

D. Effect of DMM on the glycosylation of IgM Heavy Chain

The glycosidase Endo H (streptomyces plicatus) cleaves high-mannose type oligosaccharides from glycoproteins, by hydrolysis of the glycosidic bond between the two N-acetyl glucosamine residues adjacent to the polypeptide chain. Complex and most hybrid type N-linked oligosaccharides are not hydrolysed.

Changes in the glycosylation of glycoproteins can be monitored by analysis of apparent molecular weight (Mr) changes by SDS PAGE. IgM molecules can be reduced by treatment with 2-mercaptoethanol and the Mr of the separated heavy (μ) and light (κ or λ) chains determined using SDS-PAGE by comparison with standards.

Figure 4 shows a western blot of a 10% polyacrylamide gel to specifically locate the IgM μ chains. Native BRIC 30 μ chain had a Mr of 86K and migrated as a sharp band (lane a). BRIC 30 μ chain from a culture treated with 5μg/ml DMM (lane b) had a Mr of 83.5K, 2.5K lower than native, indicating altered glycosylation.

Both native and DMM treated BRIC 30 were sensitive to Endo H digestion. Endo H (100 mU/ml) treated native BRIC 30 migrated as a broad band with a Mr of 79.5K, a reduction

of up to 6.5K from native BRIC 30 (lane c). This suggests native BRIC 30 μ chain contains some Endo H sensitive high mannose glycans. In MOPC104E Anderson et al described such glycans on residue Asn 563.

Endo H treated DMM treated BRIC 30 μ chain migrated as a sharp band with a Mr of 77.5K, a reduction of 8.5K on the native BRIC 30 μ chain and 7.0K on the DMM treated BRIC 30 μ chain (lane d).

These results indicate that the DMM treatment results in a marked increase in the susceptibility of BRIC 30 μ chain to Endo H digestion. This would be expected if DMM caused an increase in high mannose type glycans in the μ chain. These changes are concomitant with the changes in low temperature solubility of BRIC 30. Endo H could be used in this way to monitor the effectiveness of inhibitor treatment.

E. Effect of alpha - glactosidase on cryoprecipitation of BRIC 30

BRIC 30 IgM purified by ammonium sulphate precipitation was treated with alpha galactosidase and assayed for cryoprecipitation as described in Materials and Methods. Alpha galactosidase treatment of BRIC 30 was found to abolish the cryoprecipitation effect (Table 2).

When alpha galactosidase was added to cultures of BRIC 30 hybridoma cells, the cryoprecipitation of the resultant IgM was inhibited in a dose - dependenent manner (Table 3)

F. Properties of other IgM anti-B monoclonals

A number of other hybridoma clones secreting IgM

anti-B (not related to BRIC 30) were assayed for the ability to agglutinate red cells of weak B group (B3, Bcord) and also susceptibility to cryoprecipitation. The clones isolated had different properties. In general those that reacted most strongly with weak B red cells showed the greatest amount of cryoprecipitation.

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Table 1

Concentration of BRIC 30 IgM (µg/ml) in samples stored at 4°C

x2 : Material soluble at 4°C stored for a further 24 hr at 4°C.

Initial concentration	71	
Supernatant - 4°C	14	(20%)
Precipitate - 4°C	54	(80%)
Supernatant - 4°C x2	16	(97%)
Precipitate - 4°C x2	0.5	(3%)

Table 2

Effect of alpha galactosidase treatment of purified BRIC 30 IgM on cryoprecipitation

	4°C		Room Temperature	
	%S	%P	%S	%P
untreated	15	85	100	0
alpha glactosidase treated	100	0	100	0

%S: % IgM in supernatant after storage 72hr.
%P: % IgM in precipitate after storage 72hr.

Table 3

Effect of alpha galactosidase added to BRIC 30 hybridoma cell cultures on the cryoprecipitation of the resultant IgM

Concentration of alpha galactosidase µg/ml	% BRIC 30 IgM soluble after 4°C storage for 72hr
0	24
0.05	23
0.5	25
5.0	78

CLAIMS

1. Modified forms of antibodies having specifities against polysaccharide antigenic determinants which antibodies in the native form produce a precipitate on storage at 4°C in aqueous solution said modified antibodies carrying modified glycosyl groups and on storage in aqueous solution at 4°C producing substantially no precipitate.

2. Modified forms of antibodies as claimed in claim 1 comprising a mixture of antibodies each having the same polypeptide moiety but carrying different glycosyl groups, the mixture in the native form producing a precipitate at 4°C in aqueous solution, the modified antibodies carrying modified glycosyl groups whereby on storage in aqueous solution at 4°C, the mixture does not form a precipitate.

3. Modified antibodies as claimed in claim 1 or claim 2 being monoclonal antibodies.

4. Modified antibodies as claimed in any one of claims 1 to 3 being of the IgM sub-class.

5. Modified antibodies as claimed in any one of claims 1 to 4 being directed against the B blood group antigen.

6. Modified antibodies as claimed in any one of claims 1 to 5 wherein the modified glycosyl groups are free from regions cross-linking with the antigen binding sites of the antibodies.

7. A process for the preparation of modified antibodies according to any one of claims 1 to 6 wherein the antibodies are subjected to the action of one or more glycosidases.

8. A process for the preparation of modified monoclonal antibodies according to any one of claims 3 to 6 wherein the cell line producing said monoclonal antibodies in unmodified form is cultured in the presence of one or more inhibitors of glycoprotein processing.

9. A process as claimed in claim 8 wherein said inhibitor is castanospermine, deoxymannojirimycin, swainsonine or deoxynorjirimycin.

10. A method of blood typing wherein a modified antibody according to claim 5 is used.

FIG. 1

EFFECT OF IgM CONCENTRATION ON SOLUBILITY OF BRIC 30 AT 4C.

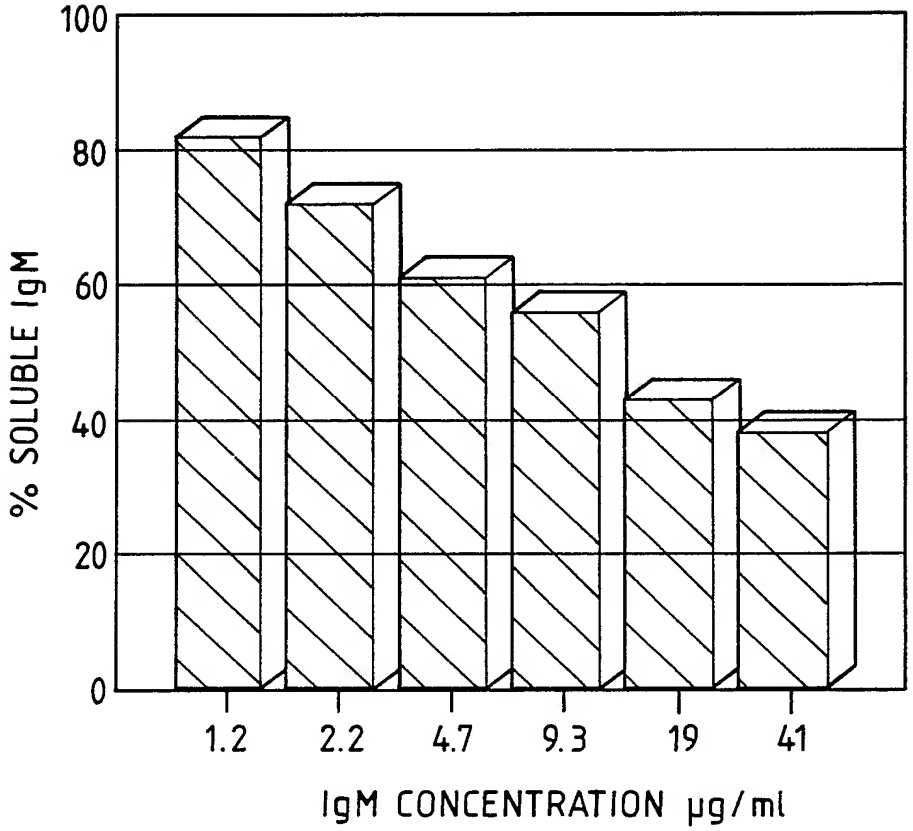


FIG. 2

EFFECT OF INHIBITORS ON SOLUBILITY OF BRIC 30 AT 4C.

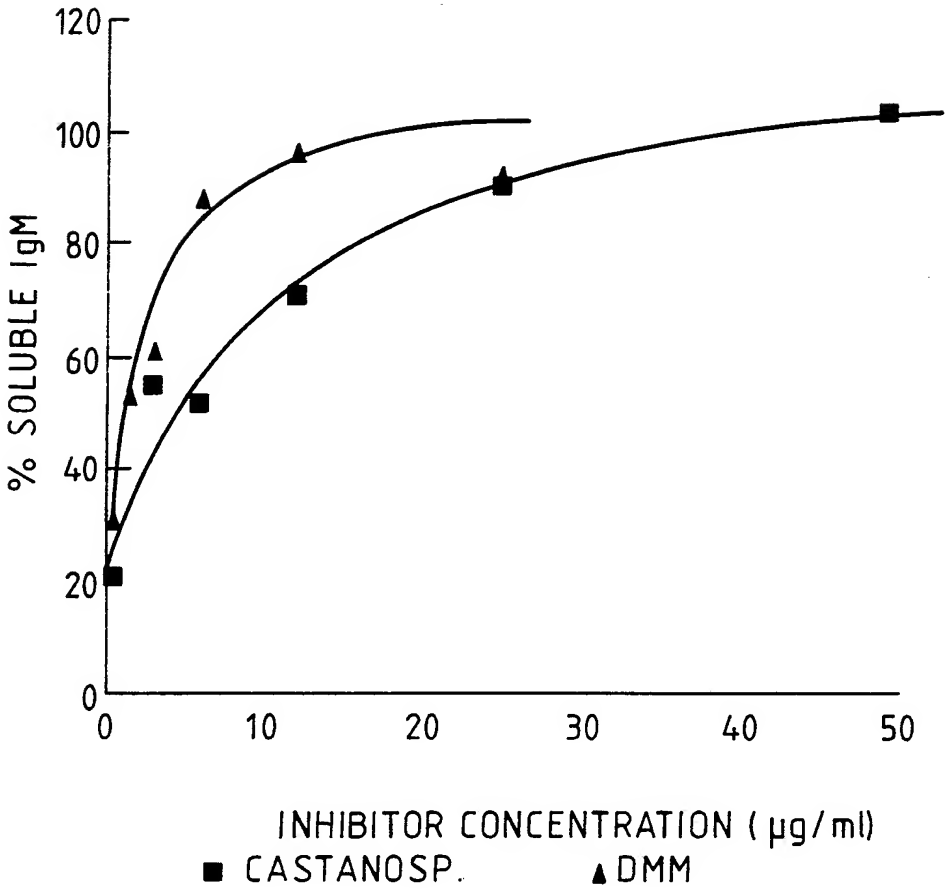


FIG. 3
EFFECT OF INHIBITORS ON YIELD OF SOLUBLE BRIC 30.

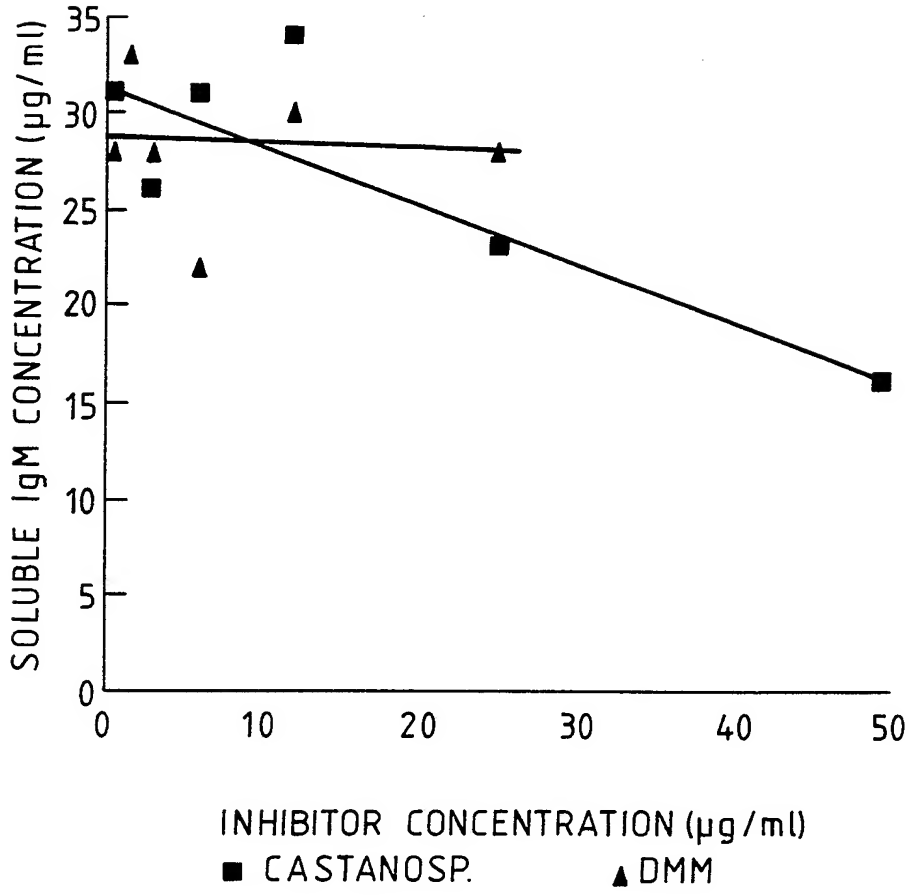
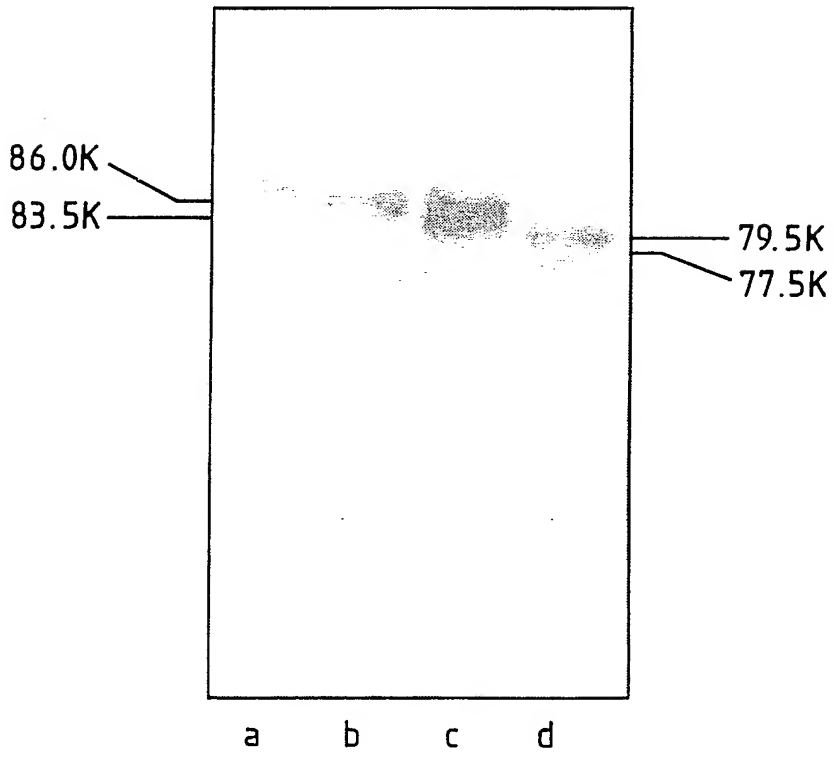


FIG . 4

EFFECT OF DMM AND OF ENDO H ON THE Mr OF BRIC 30 μ CHAIN



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/00921

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 P 21/08

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 P, C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
	No relevant documents have been disclosed.	

* Special categories of cited documents: [10]

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